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## Loss of HIF1A From Pancreatic Cancer Cells Increases Expression of PPP1R1B and Degradation of p53 to Promote Invasion and Metastasis

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### Abstract

**Background & Aims:** Pancreatic ductal adenocarcinomas (PDACs) are hypovascular, resulting in the upregulation of hypoxia inducible factor 1 alpha (HIF1A), which promotes survival of cells

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#### Author Contributions

A.T., P.P.J. and A.B. conceived and designed the overall project. A.T., K.T., A.D., performed experiments and/or analysis. K.V., B.H., M.C. performed cell invasion and migration experiments, in vivo lung colonization imaging and shRNA experiments. A.S., I.S., J.S., N.P., A.L., E. S., and U.P. participated in genotyping, cell line preparation and provided assistance with tissue culture and QPCR experiments. G.G. performed histological analysis. A.R.C performed the LC-MS/MS and Network and Pathway Analysis. B.H and A.J.D assisted with TCGA data analysis. B.H. provided all the administrative, technical and material support. T.K.S., A.J.D., C.C. and P.P.J. provided overall input in writing, review, and/or revision of the manuscript. A.T., Y.M. and A.B. wrote the manuscript. All authors reviewed and edited the manuscript. A.B oversaw all aspects of the study.

**Conflicts of interest:** The authors disclose no conflicts.

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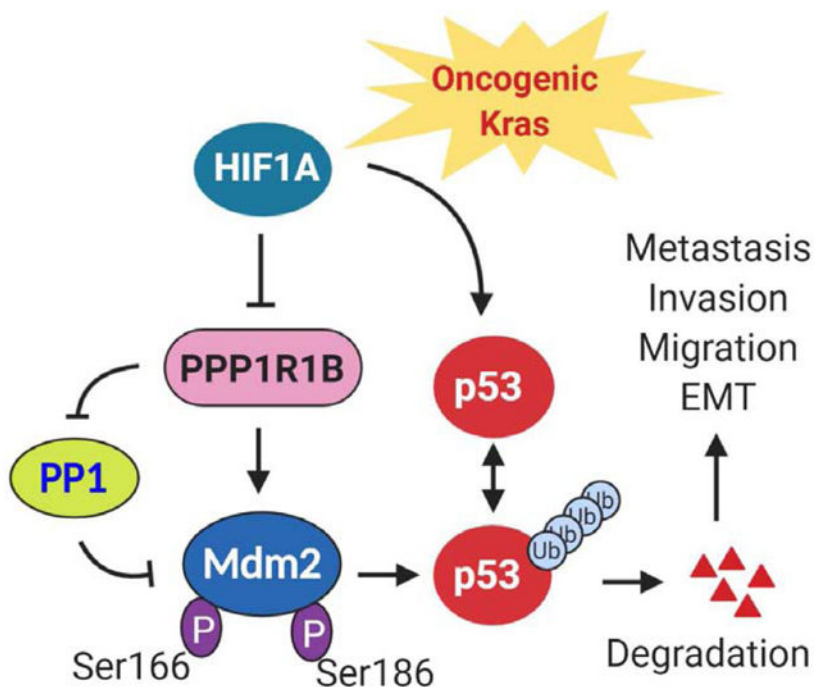
under low-oxygen conditions. We studied the roles of HIF1A in development of pancreatic tumors in mice.

**Methods:** We performed studies with  $Kras^{LSL-G12D/+}; Trp53^{LSL-R172H/+}; Pdx1-Cre$  (KPC) mice, KPC mice with labeled pancreatic epithelial cells (EKPC), and EKPC mice with pancreas-specific depletion of HIF1A. Pancreatic and other tissues were collected and analyzed by histology, and immunohistochemistry. Cancer cells were cultured from PDACs from mice and analyzed in cell migration and invasion assays and by immunoblots, real-time PCR, and liquid chromatography–mass spectrometry. We performed studies with the human pancreatic cancer cell lines PATU-8988T, BxPC-3, PANC-1 and MiaPACA-2, which have no or low metastatic activity, and PATU-8988S, AsPC-1, SUIT-2 and Capan-1, which have high-metastatic activity. Expression of genes was knocked down in primary cancer cells and pancreatic cancer cell lines using small hairpin RNAs; cells were injected intravenously into immune-competent and NOD/SCID mice and lung metastases were quantified. We compared levels of mRNAs in pancreatic tumors and normal pancreas in the Cancer Genome Atlas.

**Results:** EKPC mice with pancreas-specific deletion of HIF1A developed more advanced pancreatic neoplasias and PDACs with more invasion and metastasis, and had significantly shorter survival times, than EKPC mice. Pancreatic cancer cells from these tumors had higher invasive and metastatic activity in culture than cells from tumors of EKPC mice. HIF1A-knockout pancreatic cancer cells had increased expression of protein phosphatase 1 regulatory inhibitor subunit 1B (PPP1R1B). There was an inverse correlation between levels of HIF1A and PPP1R1B in human PDAC tumors; higher expression of PPP1R1B correlated with shorter survival times of patients. Metastatic human pancreatic cancer cell lines had increased levels of PPP1R1B and lower levels of HIF1A compared with non-metastatic cancer cell lines; knockdown of PPP1R1B significantly reduced the ability of pancreatic cancer cells to form lung metastases in mice. PPP1R1B promoted degradation of p53 by stabilizing phosphorylation of MDM2 at Ser166.

**Conclusions:** HIF1A can act a tumor suppressor by preventing expression of PPP1R1B and subsequent degradation of the p53 protein in pancreatic cancer cells. Loss of HIF1A from pancreatic cancer cells increases their invasive and metastatic activity.

## Graphical Abstract



**Keywords**

Tumor Progression; Cell Motility; Signal Transduction; Carcinogenesis

**Introduction:**

Pancreatic ductal adenocarcinoma (PDAC) is one of the most lethal human cancers becoming the fourth-leading cause of cancer-related death in the US with a 5-year survival of only 8%.<sup>1</sup> PDAC often develops without early symptoms, and therefore most patients are diagnosed with metastatic disease, a point where chemotherapeutic or surgical intervention have minimal benefit. Unlike the comprehensive understanding of genetic mutations that initiate PDAC, the molecular mechanisms that promote the metastatic spread of pancreatic cancer are less clear. In addition to the high prevalence of KRAS mutations<sup>2</sup>, PDACs are characterized by extensive desmoplastic stroma and severe hypovascularity<sup>3,4</sup> resulting in extreme intra-tumoral hypoxic microenvironment.<sup>5</sup> In response to hypoxic stress, cancer cells set off many adaptive responses through the stabilization and activation of the hypoxia inducible factor (HIF) family of transcription factors.<sup>6</sup> In PDAC, hypoxia and consequent HIF1A stabilization have been associated with the transcriptional activation of multiple pathways, involved in the regulation of metabolism, angiogenesis, cell survival, and inflammation.<sup>7,8,9</sup> Elevated HIF1A expression is usually linked with higher patient mortality in many cancer types, including breast and colorectal cancers.<sup>10,11</sup> However, emerging evidences propose that HIF1A may not be universally tumor promoting. In certain malignancies, HIF1A appears to have a tumor suppressive role and correlates with lower cancer or diminished patient mortality, implicating contrasting, context-dependent functions for HIF1A.<sup>12,13,14</sup>

The role of HIF1A in pancreatic cancer has been evolving in the recent years. Previously published reports showed inconsistent results on associations between expression of HIF1A and clinical outcomes in PDAC.<sup>15,16,17</sup> Therefore, the precise role of HIF1A in PDAC pathogenesis is not completely comprehended. In part, a lack of detailed understanding of HIF1A's role in PDAC is due to use of RNA interference of HIF1A in *in vitro* assays or xenograft tumor models,<sup>16,18</sup> which cannot truly recapitulate the complex relationship of neoplastic epithelial cells with their microenvironment. A previous study, using p48-Cre; LSL-KrasG12D mouse model of PDAC showed that HIF1A deficiency promotes PDAC initiation through enhanced B cell infiltration.<sup>14</sup> In the present study, we sought to identify the functional role of HIF1A in pancreatic cancer using a well characterized autochthonous model of PDAC that harbors the lox-stop-lox (LSL)-KrasG12D; LSL-p53R172H and Pdx-Cre (KPC) alleles that faithfully recapitulates all of the extant features of the human PDAC.<sup>19</sup> We demonstrate that ablation of HIF1A in these mice resulted in drastically increased invasion, metastasis and substantially reduced survival. We further uncovered that loss of HIF1A leads to increased expression of PPP1R1B and subsequent degradation of the p53 protein that promotes invasion and metastasis in both mouse and human PDACs.

## Material and Methods

### Mouse Strains

Kras<sup>LSL-G12D/+</sup>; Trp53<sup>LSL-R172H/+</sup>; Pdx1-Cre (KPC) mice have been previously described in detail<sup>19</sup>. ROSA26<sup>LSL-EYFP</sup> mice<sup>20</sup> (obtained from Jackson Laboratory) were crossed with KPC mice to label pancreatic epithelial cells expressing Cre (EKPC). Pancreas specific depletion of HIF1A was attained by crossing EKPC with HIF1A<sup>flox/flox</sup> conditional strain<sup>21</sup> referred as HIF1A<sup>-/-</sup>EKPC.

### Cell Lines

Mouse pancreatic cancer cells (EKPC and HIF1A<sup>-/-</sup>EKPC) were grown in DMEM with supplements as previously described.<sup>22</sup> Human pancreatic cancer cell lines were grown in their required growth medium as per the ATCC description (SI Materials and Methods).

### *In Vivo* lung colonization assay

For lung colonization assays, mouse and human pancreatic cancer cells were stably transduced with luciferase transgene injected intravenously into the tail vein of C57BL/6 mice and bioluminescence imaging was initiated on day seven post transplantation.

### LC-MS/MS and Data Analysis

Proteomics analysis was performed by the Proteomics Core, Sanford Burnham Prebys Medical Discovery Institute and data were analyzed as described in SI Materials and Methods.

### Human Data Sets

The gene expression data and survival analyses of PDAC patients with annotated clinical outcomes were downloaded from The Cancer Genome Atlas. We used cBioPortal<sup>23, 24</sup> and

GEPIA<sup>25</sup> for data visualization, analysis and download of large-scale cancer genomics data sets.

### Quantification and Statistical Analysis

Statistical analyses for figures were performed using GraphPad Prism software. Data are presented as the mean  $\pm$  s.e.m unless otherwise specified. All experiments were performed at least three independent times, unless otherwise noted. A log-rank (Mantel-Cox) test was used to evaluate statistical significance for the Kaplan-Meier survival plots.

## Results

### Loss of HIF1A leads to adverse outcome in PDAC

To study the role of HIF1A in PDAC *in vivo*, we used the KPC mouse, a well characterized autochthonous model of PDAC that harbors the lox-stop-lox (LSL)-KrasG12D; LSL-p53R172H and Pdx-Cre (KPC) alleles.<sup>19</sup> Additionally, we introduced an LSL-Rosa-EYFP allele<sup>20</sup> into this model to facilitate identification of the carcinoma cells (EKPC). Finally, we added a conditional allele of HIF1A<sup>21</sup> to generate murine PDACs lacking HIF1A (HIF1A<sup>-/-</sup>EKPC, Figure 1A, Supplementary Figure 1A-E). Loss of HIF1A expression was confirmed by immunohistochemistry of PDACs obtained from HIF1A<sup>-/-</sup>EKPC mice. (Supplementary Figure 1F). Since HIF1A is most commonly thought to contribute to poor outcome in cancer patients,<sup>10,11, 26, 27</sup> we expected HIF1A<sup>-/-</sup> EKPC mice to have diminished disease burden and better survival rate than the EKPC cohort. Surprisingly, HIF1A<sup>-/-</sup>EKPC mice exhibited significantly worse outcome. While the median survival of the EKPC mice (n=25 ) was 159 days, similar to that reported previously,<sup>19, 28</sup> the HIF1A<sup>-/-</sup>EKPC mice (n=24) had a significantly abbreviated median survival of 113 days (p<0.005) (Figure 1B). Comprehensive necropsy of HIF1A<sup>-/-</sup>EKPCs and EKPCs revealed more aggressive disease in HIF1A<sup>-/-</sup>EKPC mice (Table 1). Careful analysis of pancreatic intraepithelial neoplasias (PanINs) in the EKPC and HIF1A<sup>-/-</sup>EKPC mice revealed that most of the evaluable PanINs in the pancreata of HIF1A<sup>-/-</sup>EKPC mice had more diffuse boundaries that suggest more advanced PanIN lesions that have progressed closer to, but have not yet achieved, focal invasion (i.e. progression to frank PDAC), compared with more defined boundaries in the PanINs found in EKPC mice (Figure 1C). Additionally, 75% of the PDACs found in HIF1A<sup>-/-</sup>EKPC mice showed evidence of direct local invasion into the adjacent organs (i.e. locally invasive PDAC), compared with 24% of those in the EKPC cohort (Figure 1D,E, Table 1). While 60% of EKPC mice display metastatic dissemination, 91.6% of HIF1A<sup>-/-</sup>EKPCs displayed incidences of metastasis (Figure 1D,F, Table 1). Additionally, 75% of HIF1A<sup>-/-</sup>EKPC mice displayed macro metastatic lesions compared with only 36% found in the EKPC cohort (Figure 1G,H, Table 1). HIF1A<sup>-/-</sup>EKPC mice also exhibited higher percentage of lymph node metastasis and increased dissemination of metastasis in multiple organs (Figure 1I,J, Supplementary Figure 2, Table 1). We next examined the effects of HIF1A depletion on tumor differentiation. Although there was no significant difference in overall histological grades between the EKPC and HIF1A<sup>-/-</sup>EKPC PDACs, we observed higher percentage of PanINs in HIF1A<sup>-/-</sup>EKPC tumors compared to EKPC tumors, indicating that the loss of HIF1A facilitates the initiation and progression of PDAC precursors (Supplementary Figure 3). To gain insight into how HIF1A modulates

intra-tumoral hypoxia and stroma during the PDAC development, we performed immunohistochemical staining for carbonic anhydrases-9 (a surrogate marker of tumor hypoxia) and stromal contents including Collagen type I, type III and  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA). IHC staining and histologic analysis showed no difference in these markers between EKPC and HIF1A<sup>-/-</sup>EKPC PDAC sections indicating that HIF1A is largely dispensable for the intra-tumoral stromal content in mouse PDAC. (Supplementary Figure 4). We further analyzed the composition of immune cell infiltration in EKPC and HIF1A<sup>-/-</sup>EKPC tumors by immunohistochemistry (Supplementary Figure 5). We observed significant increase in CD19+ B cell population in HIF1A<sup>-/-</sup>EKPC tumors, similar to an earlier report that HIF1A deficiency enhances B-cell infiltration in PDACs.<sup>14</sup> We further analyzed the effect of HIF1A inhibition after the PDACs have been established in KPC mice by using pharmacological inhibitor of HIF1A (PX-478). Based on Ki67 immunostaining, we observed pronounced increase in cell proliferation in tumor sections from KPC mice treated with HIF1A inhibitor ( $P = 0.008$ ) (Figure 1K,L), suggesting that inhibition of HIF1A either genetically or pharmacologically can promote tumorigenesis in these mice. Taken together, these data clearly demonstrate that loss of HIF1A in mouse PDACs resulted in markedly increased invasion and metastasis, with reduced survival rate.

### Depletion of HIF1A enhances invasion and migration of Pancreatic Cancer cells

To further investigate whether HIF1A impacts the invasive and the metastatic behavior of the PDACs in a cell autonomous manner, we derived multiple cell lines from primary PDACs in EKPC and HIF1A<sup>-/-</sup>EKPC mice (Supplementary Figure 6). Interestingly, while cell lines derived from EKPC primary tumors showed considerable phenotypic heterogeneity ranging from epithelial to mixed mesenchymal, all the tumor lines derived from HIF1A<sup>-/-</sup>EKPC PDACs showed consistent and noticeable mesenchymal state with elongated morphology, suggesting an invasive phenotype (Figure 2A). To better understand the role of other HIFs in the context of loss of HIF1A in PDACs, we analyzed expression of other HIFs at transcriptional and protein level in EKPC and HIF1A<sup>-/-</sup>EKPC cells, in hypoxic and normoxic conditions. As expected we observed increased mRNA and protein expression of HIF1A, HIF1B and HIF2A in the EKPC and HIF1B, and HIF2A expression in the HIF1A<sup>-/-</sup>EKPC cells under hypoxic conditions (Figure 2B,C). We did not detect any expression of HIF3A transcripts in normoxic or hypoxic conditions in any of these cells lines. However, we did not observe any compensatory increase in HIF1B and HIF2A in HIF1A<sup>-/-</sup>EKPC cells (Figure 2B). Furthermore, we analyzed expression of HIF1A and HIF2A in both EKPC and HIF1A<sup>-/-</sup>EKPC PDACs by immunohistochemistry. Consistent with our cell line data, we did not observed any significant increase in HIF2A expression in PDACs derived from HIF1A<sup>-/-</sup>EKPC mice (Figure 2D,E). Next, we examined the invasive and migratory capacity of EKPC and HIF1A<sup>-/-</sup>EKPC cells by *in vitro* transwell matrigel assays. We observed that HIF1A<sup>-/-</sup>EKPC cells manifested significantly higher invasion and migration compared with the EKPC cells (Figure 2F,G). Epithelial–mesenchymal transition (EMT) has been shown to play a critical role in promoting metastasis in carcinomas. Indeed, HIF1A<sup>-/-</sup>EKPC cells exhibited elevated EMT, including reduced expression of the epithelial marker E-Cadherin and increased expression of the mesenchymal markers Snail and Slug at the transcriptional as well as the protein levels (Figure 2H, Supplementary Figure 7A). We further investigated the effect of HIF2A on EMT regulators in HIF1A<sup>-/-</sup>EKPC cells by

knocking down its expression using siRNA. We did not observe any reduction in E-Cadherin, SNAIL and SLUG expression in HIF2A depleted HIF1A<sup>-/-</sup>EKPC cells (Supplementary Figure 7B). To investigate how EMT markers like Snail and Slug is upregulated in absence HIF1A, we found that all the three HIF1A<sup>-/-</sup>EKPC biological replicates exhibited higher expression of c-MYC protein compared to the EKPC counterparts (Figure 2G). We further investigated the role of c-MYC on Snail and Slug expression by depleting c-MYC in HIF1A<sup>-/-</sup>EKPC cells. Knockdown of c-Myc in HIF1A<sup>-/-</sup>EKPC cell lines impaired both Snail and Slug expression (Supplementary Figure 7C), indicating that the regulation of EMT markers in HIF1A<sup>-/-</sup>EKPC may be controlled by pathways independent of HIF1A, c-MYC being one of them. To further confirm that the loss of HIF1A resulted into increased EMT features in HIF1A<sup>-/-</sup>EKPC, we performed the rescue experiment by ectopically expressing a mutant version of HIF1A<sup>P402A/P577A/N813A</sup> (m-HIF1A) which is stable and active in normoxia,<sup>29</sup> which resulted in more epithelial morphology and increased E-Cadherin and decreased N-Cadherin expression in HIF1A<sup>-/-</sup>EKPC cells (Supplementary Figure 7D,E).

To investigate if HIF1A impedes metastatic potential of the PDAC *in vivo*, equal number of luciferase expressing HIF1A<sup>-/-</sup>EKPC and EKPC cells were injected via tail vein into immunocompetent C57BL/6 mice (Figure 2I) and their dissemination was evaluated at various time points by bioluminescence imaging (BLI). Mice injected with HIF1A<sup>-/-</sup>EKPC cells had significantly more luciferase signal compared to the ones injected with EKPC cells (Figure 2J,K, Supplementary Figure 8). Moreover, mice inoculated with HIF1A<sup>-/-</sup>EKPC cells had reduced survival rate compared to those injected with EKPC cells (Figure 2L). Taken together, the results suggest more efficient lung colonization by HIF1A<sup>-/-</sup>EKPC cells compared to EKPC cells. Indeed, enumeration of lung nodules in the two cohorts show HIF1A<sup>-/-</sup>EKPC cells formed significantly more tumor nodules than the EKPC cells (Supplementary Figure 8B,C), thus phenocopying the metastatic effect observed in HIF1A<sup>-/-</sup>EKPC mice. These data provide evidence that genetic inhibition of HIF1A in the pancreatic cancer cells induces EMT and significantly increases their invasive and metastatic potential in a cell autonomous manner.

### HIF1A regulates PPP1R1B in pancreatic cancer

To delineate a pathway whereby HIF1A inhibits invasion and metastasis in PDAC, we performed a comparative proteomic analysis between EKPC and HIF1A<sup>-/-</sup>EKPC cells (Figure 3A). Ingenuity Pathway Analysis (IPA) of the proteomics data revealed that the most significantly activated cellular functions in HIF1A<sup>-/-</sup>EKPC cells included cell movement ( $P=3.84E11-09-06$ ), invasion ( $P=2.15E$ ) and migration ( $P=1.73E$ ), whereas the most significantly suppressed cellular functions were cell-cell adhesion ( $P=2.67E^{-04}$ ), binding ( $P=3.03E^{-04}$ ) and cell-cell contact ( $P=1.01E^{-03}$ ) (Figure 3B, Supplementary Figure 9, Supplementary Table 1). These results further validate our conclusion that inhibition of HIF1A promotes invasion and metastasis in PDAC. Intriguingly, one of the top differentially expressed proteins was a dual kinase/phosphatase inhibitor named protein phosphatase 1 regulatory inhibitor subunit 1B (PPP1R1B) (Figure 3C, Supplementary Table 2). PPP1R1B was first shown to be expressed in the adult brain in regions that are responsive to dopamine, consistent with its alternate name Dopamine And cAMP Regulated Phosphoprotein 32

(DARPP-32).<sup>30</sup> At the mechanistic level, PPP1R1B/DARPP-32 regulates downstream signaling of a variety of kinases through its ability to bind and inhibit the activity of Protein Phosphatase 1. This, in turn, controls the activity of hundreds of phosphorylated proteins.<sup>31</sup> Increased levels of PPP1R1B transcripts have been reported in several cancers including breast, colon, esophagus, lung, and prostate<sup>32–36</sup> and elevated expression of PPP1R1B has been associated with advanced disease and poor prognosis in several cancers<sup>33, 37</sup> (Supplementary Figure 10). However, its role in PDAC and in metastasis remains poorly understood.

We observed significant upregulation of Ppp1r1b transcript and protein in HIF1A<sup>-/-</sup>EKPC cells compared to EKPC cells (Figure 3D,E) suggesting Ppp1r1b is directly regulated by HIF1A. EKPC cells treated with the hypoxia-mimetic agent CoCl<sub>2</sub> resulted into decreased expression of Ppp1r1b, but no such reduction of Ppp1r1b expression was observed in the HIF1A<sup>-/-</sup>EKPC cells (Figure 3F). Conversely, overexpression of m-HIF1A in HIF1A<sup>-/-</sup>EKPC cells resulted into reduced expression of Ppp1r1b (Supplementary Figure 11A). Immunohistochemistry using antibody against PPP1R1B revealed intense signal in the frank PDACs as well as in metastatic lesions in the HIF1A<sup>-/-</sup>EKPC mice as compared with the EKPC counterparts (Figure 3 G,H, Supplementary Figure 11B-D). To determine whether the observed inverse relationship between HIF1 $\alpha$  and PPP1R1B is true in human pancreatic cancers, we carried out similar analysis in human metastatic and non-metastatic pancreatic cancer cell lines. For our analysis, we chose the well-studied human pancreatic cancer cell lines, BxPC-3, PANC-1, MIAPaCa-2, PATU8988T (Non/low-metastatic) and AsPC-1, PATU8988S, SUIT-2 and Capan-1 (High-metastatic). Notably, PATU8988T and PATU8988S are derived from the same patient.<sup>38</sup> We performed comparative expression analysis of HIF1A and PPP1R1B in these non/low-metastatic and high-metastatic human pancreatic cancer cell lines (Figure 3I-K). Consistent with our observation, we found significant inverse correlation between HIF1A and PPP1R1B, with increased PPP1R1B and decreased HIF1A being a prominent signature in all the human pancreatic cancer cells known for high metastatic dissemination (AsPC-1, PATU8988S, SUIT-2 and Capan-1). We did not find any significant change in HIF1B, HIF2A and HIF3A expression between metastatic and nonmetastatic pancreatic cancer cells (Figure 3L). To further validate that HIF1A can directly regulate PPP1R1B expression in human pancreatic cancer cells, we carried out shRNA mediated knockdown of HIF1A in two human pancreatic cancer cell lines, PATU8988T and MIAPaCa-2. Ablation of HIF1A in these cells led to increased PPP1R1B protein levels, clearly demonstrating that HIF1A negatively regulates PPP1R1B expression in human pancreatic cancer cells (Figure 3M). We further analyzed expression of HIF1A and PPP1R1B transcripts in a dataset of human PDAC patients<sup>39</sup> obtained from the TCGA and human pancreatic cancer cell lines from CCLE database.<sup>40</sup> We found that the expression of HIF1A and PPP1R1B are inversely correlated (Pearson coefficient  $-0.184$ ;  $P=0.016$ ) (Figure 3N, Supplementary Figure 12). Further, a paired analysis among PDAC patients (n=38) ranked based on Z scores ( $> 0.5$ ) showed significant inverse relationship between PPP1R1B and HIF1A expression ( $P=0.0001$ ) (Figure 3O, Supplementary Table 3). These results suggest that PPP1R1B is regulated by HIF1A and is more abundant in the metastatic pancreatic cancer cell lines, suggesting their potential role in pancreatic cancer metastasis.



## Increased expression of PPP1R1B correlates with poor survival and its knockdown inhibits malignant properties of PDAC cells

We then investigated the clinical significance of PPP1R1B expression in patients with PDAC using the TCGA database. We found elevated expression of PPP1R1B in PDAC samples compared to normal pancreas (Figure 4A). Kaplan-Meier analysis suggested significantly better overall survival of patients with low PPP1R1B expression,  $P=0.0355$  (Figure 4B). Immunohistochemistry for PPP1R1B using human tumor microarray confirmed elevated expression of PPP1R1B in PDAC and distal metastasis compared to benign pancreas and other normal tissues (Figure 4C). We next validated the metastatic ability of PATU8988S cell line showing HIF1A<sup>lo</sup>/ PPP1R1B<sup>hi</sup> ratio as compared to its syngeneic counterpart, PATU8988T cell line exhibiting HIF1A<sup>hi</sup>/ PPP1R1B<sup>lo</sup> ratio in *in vivo* lung colonization assay (Supplementary Figure 13A-C). Following the observation that high expression of PPP1R1B promotes lung colonization of human PDAC cell lines, we investigated whether PPP1R1B is critical for accelerated metastasis in human pancreatic cancer. We knocked down PPP1R1B in PATU8988S cells using shRNA (Figure 4D). PATU8988S cells transfected with shPPP1R1B or sh.Control (sh.Ctrl) were injected intravenously into NOD/SCID mice and were subsequently assessed for lung metastasis. Whereas PATU8988S +sh.Ctrl cells formed tumor nodules in the lungs at expected frequency, PPP1R1B depletion led to significantly reduced lung metastasis (Figure 4E,F, Supplementary Figure 13D,E). This data clearly demonstrated that increased expression of PPP1R1B results into higher metastatic dissemination of pancreatic cancer cells.

## PPP1R1B promotes metastasis in pancreatic cancer by reducing p53

We next investigated the pathway through which PPP1R1B promotes metastasis in PDAC. In our proteomics analysis, we observed significantly reduced levels of p53 protein ( $-8.80$  log<sub>2</sub>fold change) in HIF1A<sup>-/-</sup>EKPC cells compared to EKPC cells (Figure 5A, Supplementary Table 2), indicating that p53 protein levels are HIF1A dependent in EKPC tumors. P53 is known to be stabilized by HIF1A.<sup>41</sup> Western blot analysis revealed reduced p53 protein in the HIF1A<sup>-/-</sup>EKPC cells as well as in HIF1A<sup>lo</sup>/ PPP1R1B<sup>hi</sup> cancer cell lines, AsPC-1 and PATU8988S (compared to HIF1A<sup>hi</sup>/ PPP1R1B<sup>lo</sup> cells) (Figure 5B,C). Since the reduction or loss of p53 protein was evident in the cells with high levels of PPP1R1B, we investigated whether elevating PPP1R1B expression reduces p53 levels. Ectopic expression of PPP1R1B in HIF1A<sup>hi</sup>/ PPP1R1B<sup>lo</sup> cell lines (BxPC-3 and PATU8988T), resulted into reduced p53 protein compared to the control (Figure 5D). Next, we wanted to investigate how expression of PPP1R1B may reduce p53 protein. Intracellular p53 levels are regulated by the E3 ubiquitin ligase, MDM2.<sup>42</sup> Phosphorylation of MDM2 at Ser166 maintains its stability by preventing self-ubiquitination.<sup>43</sup> De-phosphorylation of MDM2<sup>Ser166</sup> is critical for its degradation and concomitant stability of p53 (Figure 5E). We hypothesized that PPP1R1B, a protein phosphatase 1 inhibitor, can stabilize MDM2 by inhibiting dephosphorylation of MDM2<sup>Ser166</sup> and thereby effecting p53 degradation. Ectopic expression of PPP1R1B should then increase the phosphorylation of MDM2<sup>Ser166</sup>. Indeed, expression of PPP1R1B increased the phosphorylation of MDM2<sup>Ser166</sup> in PATU8988T and BxPC-3 (Figure 5F), suggesting that PPP1R1B potentiates MDM2 via increased MDM2<sup>Ser166</sup> phosphorylation, which in turn leads to less stable p53 protein. The effect of PPP1R1B on p53 stability was measured in both PATU8988T and BxPC-3 cell lines by

overexpressing PPP1R1B and performing a cycloheximide chase assay. The stability of p53 protein was significantly less ( $P < 0.05$ ) in PPP1R1B overexpressing PATU8988T and BxPC-3 cells (Figure 5G,H and Supplementary Figure 14A,B), thus suggesting that increased PPP1R1B expression in PDAC cells results in p53 degradation.

Based on these observations, we hypothesized that PPP1R1B mediated loss of residual p53 in the PDACs leads to increased metastatic potential of these cells. To test this, we sought to determine whether loss of p53 in EKPC cells enhanced their metastatic ability, as observed in HIF1A<sup>-/-</sup> EKPC cells (Figure 2I,J). We knocked down p53 in EKPC cell lines by stably expressing shRNA against p53 (Figure 5I). Equal number of EKPC+shp53 and EKPC +sh.Ctrl cells were injected into the lateral tail vein of immunocompetent C57/BL6 mice, and their lung colonization was evaluated at various times by bioluminescence. Loss of residual p53 in the EKPC cells resulted in a significant increase in metastatic potential compared to the control cells (Figure 5J, K). Taken together, these results support a model in which depletion of HIF1A promotes metastasis in pancreatic cancer cells, in part, via de-regulation of PPP1R1B, leading to increased phosphorylation at the MDM2<sup>Ser166</sup> and reduction of p53 in pancreatic cancer (Figure 5L).

## Discussion

In spite of the expression of pro-angiogenic factors, pancreatic cancer remains persistently hypo vascular. This results in acute hypoxia and upregulation of HIF1A, a key transcription factor responsible for inducing changes in the tumor metabolism that allows it to adapt to insufficient oxygen.<sup>44</sup> Hypoxia has been shown to induce the Hedgehog signaling,<sup>16</sup> stimulate cancer-associated fibroblasts<sup>45-47</sup> and has been suggested to contribute to poor outcome in several cancers, including PDAC.<sup>48-50</sup> Thus, there have been considerable efforts to understand and target HIF1A for clinical intervention, especially in hypoxic cancers like PDAC.<sup>7</sup>

Our results, however, support the emerging evidence that the role of HIF1A is not universally oncogenic. The ability of HIF1A to function as a tumor suppressor was first shown in VHL-deficient renal carcinoma, where patients harboring a focal deletion of HIF1A at chromosome 14q presented a more aggressive form of the cancer.<sup>12</sup> Subsequently, tumor suppressive role of HIF1A has also been shown in acute myeloid leukemia and IDH-mutant gliomas.<sup>13,51</sup> Genetic deletion of HIF1A has also been shown to promote pro-tumorigenic role for B cells in a pancreatic cancer model.<sup>14</sup> Additionally, Leppänen *et al.* demonstrated that weak HIF1A expression correlated with poor prognosis in resectable PDAC.<sup>17</sup> These studies, along with the results reported here, suggest that the role of HIF1A in cancer is context dependent. Although in some cases it appears to promote tumorigenesis, loss of HIF1A seems to drive a more malignant phenotypes in other cases. Here, we explored the functional consequences of the loss of HIF1A in pancreatic cancer and provide multiple lines of evidence that the deficiency of HIF1A promotes invasive and metastatic phenotype of PDAC cells. These results have great significance in our understanding of HIF1A as well as the potential treatment of pancreatic cancer.

Enhanced expression of other HIFs due to loss of HIF1A expression, mostly HIF2A, has been reported in some malignancies, particularly in renal cancer.<sup>52</sup> However, we did not observe any compensatory increase in HIF2A in HIF1A<sup>-/-</sup>EKPC cells. A number of other studies also stated no such compensatory link between HIF1A and HIF2A.<sup>53</sup> Loss of HIF1A have also been reported to result in enhanced cell proliferation during KrasG12D-driven PanIN progression, but no apparent compensatory role of HIF2A was observed.<sup>14</sup> Taken together, these data suggest that differential activation of HIF2A and/or other HIFs in the context of tumor progression due to loss of HIF1A may be dependent upon the mutational landscape of the tumor cells, cell types and the stability of other cofactors.<sup>54</sup> However, in order to address a role of HIF2A in the context of HIF1A deficient PDAC in an unambiguous manner, an autochthonous mouse model of PDAC needs to be studied in which both HIF1A and HIF2A can be genetically manipulated.

This study also identified PPP1R1B, a gene negatively regulated by HIF1A, as a key promoter of metastasis in pancreatic cancer. Elevated expression of PPP1R1B have been associated with advanced disease and poor prognosis in several cancers including breast and lung cancer.<sup>33-35</sup> Also in our analyses, elevated levels of PPP1R1B correlated with poorer outcome in pancreatic cancer patients. We also found inverse correlation between HIF1A and PPP1R1B, (high PPP1R1B and low HIF1A levels) being a prominent signature in several human pancreatic cancer cells known for high metastatic dissemination. We show that genetic inhibition of PPP1R1B in human pancreatic cancer cells significantly reduces their invasive and metastatic spread *in vivo*.

We also noticed a significant reduction in p53 protein stability in HIF1A compromised human and mouse PDACs. Earlier studies have suggested that HIF1A can stabilize p53.<sup>41, 55</sup> We propose that stabilization of p53 by HIF1A can be mediated by HIF1A's ability to down-regulate PPP1R1B. Loss of HIF1A and concomitant de-regulation of PPP1R1B results in loss of p53 in pancreatic cancer cells. Our data clearly suggest that loss of residual p53 in PDAC, which may include a mutant p53 allele, can lead to a significant increase in the metastatic potential of pancreatic cancer cells. This observation is in line with recent studies which implicate loss of p53 as a major driver of metastasis in KRas driven cancers.<sup>56-58</sup> Finally, inactivation of protein phosphatases, namely PP1 and PP2A, has been shown to be prevalent in several cancers, including PDACs.<sup>59, 60</sup> Activation of these protein phosphatases by exploiting their regulators are emerging as possible therapeutic intervention for these cancers.<sup>61, 62</sup> Our study identifies PPP1R1B as a potential target to mitigate pancreatic cancer metastasis and provides a detailed molecular understanding of how inhibiting HIF1A may influence the outcome of pancreatic cancer unfavorably in clinical setting.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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**What you need to know:****Background and Context:**

Pancreatic ductal adenocarcinomas (PDACs) are hypovascular, resulting in the upregulation of hypoxia inducible factor 1 alpha (HIF1A), which promotes survival of cells under low-oxygen conditions. We studied the roles of HIF1A in development of pancreatic tumors in mice.

**New Findings**

HIF1A can act a tumor suppressor by preventing expression of PPP1R1B and subsequent degradation of the p53 protein in pancreatic cancer cells. Loss of HIF1A from pancreatic cancer cells increases their invasive and metastatic activity.

**Limitations**

This study was performed in mice and human cell lines and tissues. Further studies are needed of this pathway in human pancreatic tumor progression.

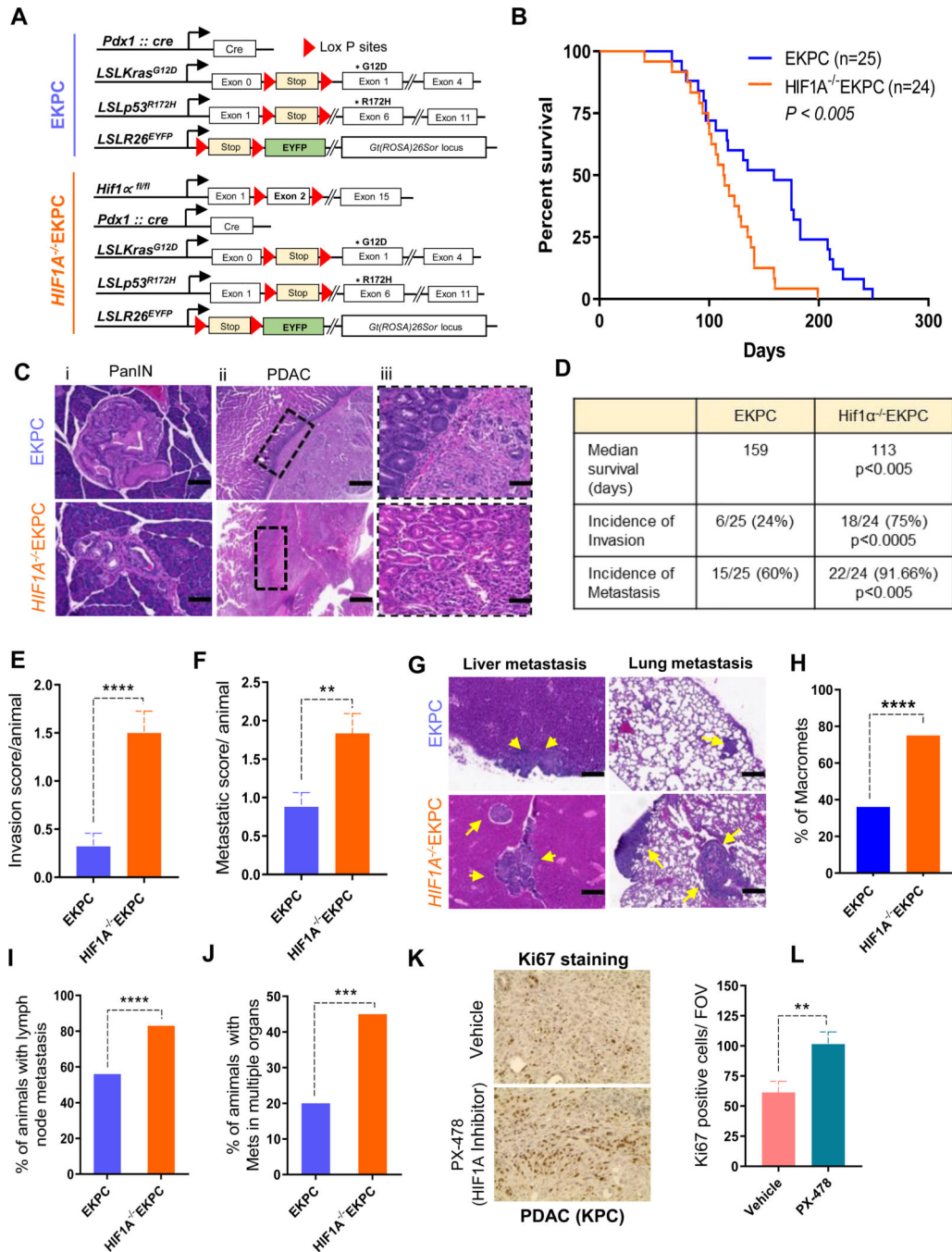
**Impact**

HIF1A is a tumor suppressor in pancreatic cancer cells; strategies to restore its activity might be developed for treatment of PDAC.

**Lay Summary**

The researchers identified a protein that is lost from pancreatic cancer cells that allows them to become more invasive and promote tumor metastasis.

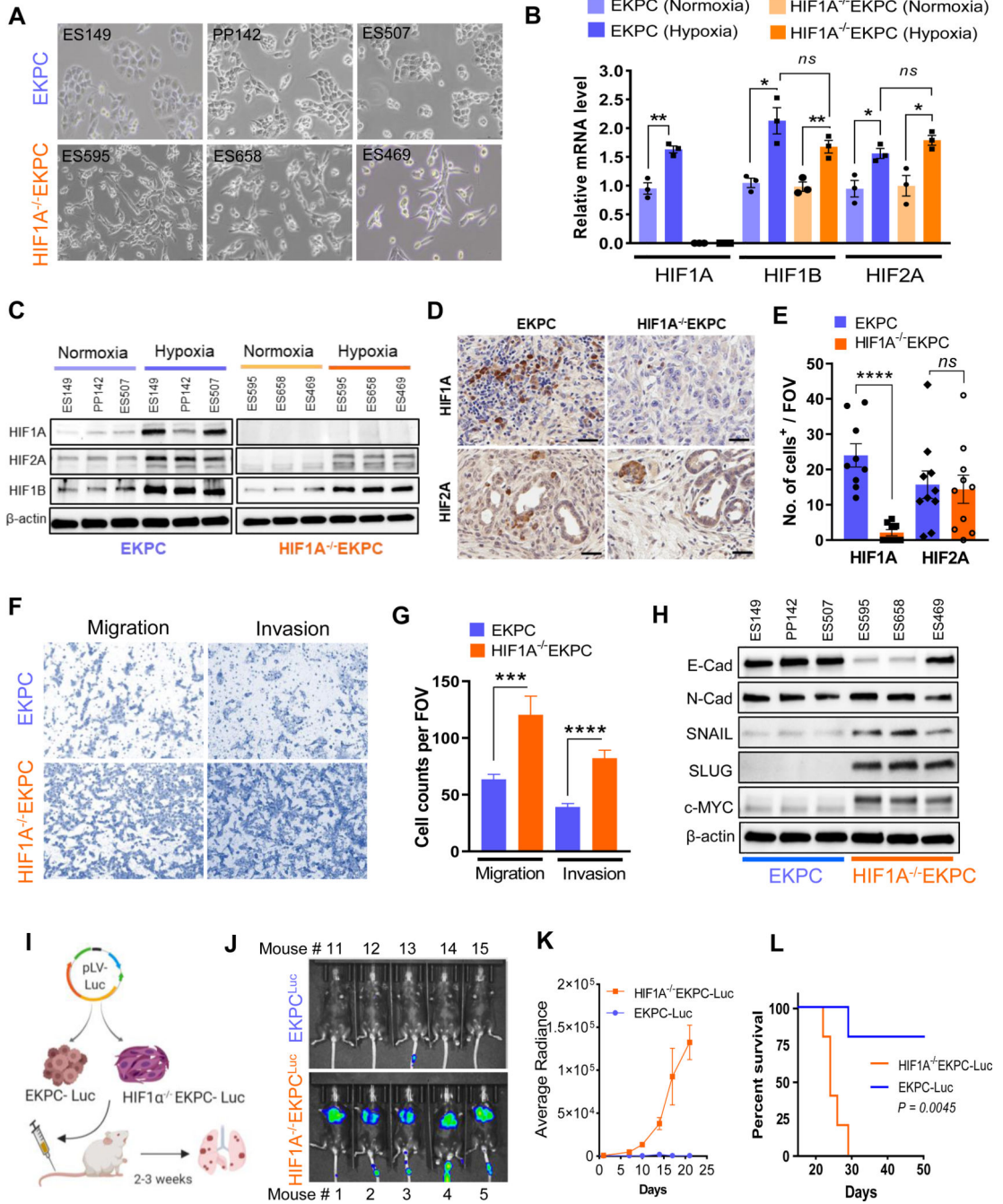




**Figure 1: Loss of HIF1A leads to adverse outcome in PDAC.**

(A) Schematics of EKPC (Blue) mouse model of pancreatic cancer, which employs *LSLKras<sup>G12D/+</sup>* (K), *LSLp53<sup>R172H/+</sup>* (P), *Pdx1-Cre* (C), and *LSLRosa EYFP* (E) alleles. Pancreas specific depletion of HIF1A was attained by crossing EKPC with *HIF1A<sup>fl/fl</sup>* mouse strain and referred as HIF1A<sup>-/-</sup>EKPC (Orange). (B) Kaplan-Meier analysis comparing survival of EKPC (n = 25) and HIF1A<sup>-/-</sup>EKPC (n = 24) mice; \*\**P*<0.005 Mantel-Cox test. (C) Hematoxylin and eosin (H&E) staining of pancreata from EKPC and HIF1A<sup>-/-</sup>EKPC mice. Loss of HIF1A promotes more advanced PanINs and locally invasive

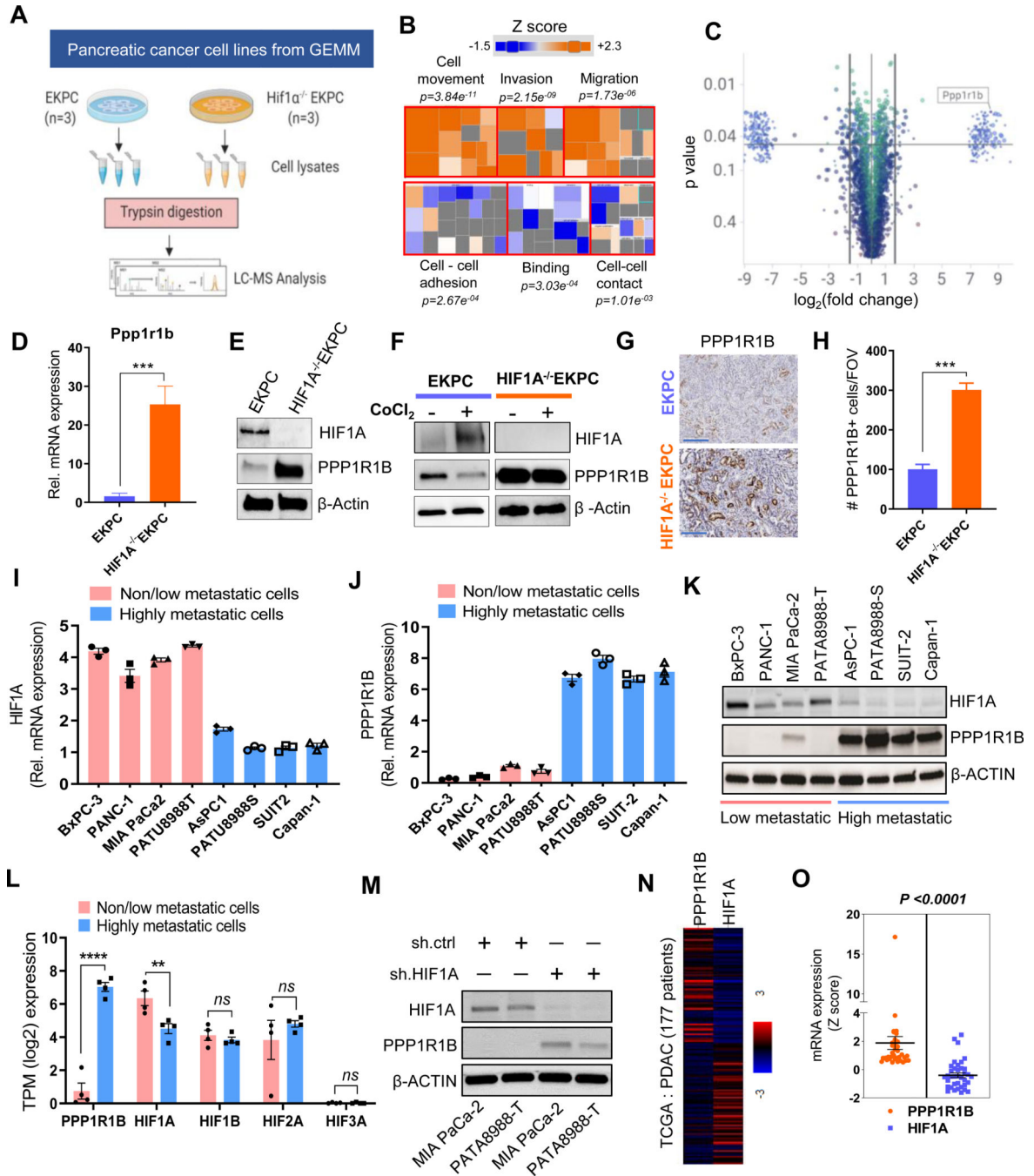
and metastatic PDAC. Representative PanIN lesions in EKPC and HIF1A<sup>-/-</sup>EKPC mice (i). The duodenum-PDAC interface in EKPC and HIF1A<sup>-/-</sup>EKPC mice showing a distinct example of an intact boundary without local invasion in EKPC mice, whereas PDACs from HIF1A<sup>-/-</sup>EKPC mice frequently show local invasion into adjacent organs (ii & iii) **(D)** Table showing comparative median survival ( $P<0.005$ ), and incidences of local invasion ( $P<0.0005$ ) and metastasis ( $P<0.005$ ) in EKPC and HIF1A<sup>-/-</sup>EKPC mice; by Fisher's exact test. **(E)** Comparative Invasion score (total number of organs showing local invasion by primary tumor divided by total number of animals) in EKPC (n=25) and HIF1A<sup>-/-</sup>EKPC mice (n=24), \*\*\*\* $P<0.0001$  by unpaired t test **(F)** Metastatic score (total number of organs showing Mets divided by total number of animals) in EKPC (n=25) and HIF1A<sup>-/-</sup>EKPC mice (n=24), \*\*  $P<0.005$  by unpaired t test **(G)** Representative H&E stained images of the liver and lung mets (marked by arrows) in EKPC and HIF1A<sup>-/-</sup>EKPC cohorts. (Scale bar:10 $\mu$ m C,F) **(H)** Percentage of Macro-mets in EKPC and HIF1A<sup>-/-</sup>EKPC, \*\*\*\* $P<0.0001$  by Fisher's exact test **(I)** Percentage of EKPC and HIF1A<sup>-/-</sup>EKPC mice with lymph node involvement; \*\*\*\* $P<0.0001$  by Fisher's exact test **(J)** Percentage of EKPC and HIF1A<sup>-/-</sup>EKPC mice with metastasis in more than one organ; \*\*\* $P<0.001$  by Fisher's exact test. **(K)** Representative Ki-67 staining for proliferating cells in PDAC from KPC mice treated with HIF1A inhibitor (PX-478) and vehicle control **(L)** Quantification of Ki-67 staining as total number of cells per field of vision in tumor sections from control and treated KPC mice. 10 FOV from each treatment group were randomly chosen and Ki67 positive cells were calculated, \*\* $P<0.005$  by unpaired t test.



**Figure 2: Depletion of HIF1A enhances invasion and migration of Pancreatic Cancer cells.**

(A) Bright field image of primary cell lines derived from EKPC (ES149, PP142 & ES507) and HIF1A<sup>-/-</sup> EKPC (ES595, ES658 & ES469) tumors. (B) Expression of HIF1A, HIF1B, and HIF2A mRNA under hypoxic/normoxic conditions in EKPC and HIF1A<sup>-/-</sup> EKPC cell lines. Relative gene expression level for HIF1A, HIF1B, and HIF2A in EKPC (blue) and HIF1A<sup>-/-</sup> EKPC (orange) cells were normalized with GAPDH expression; n=3 biologically independent experiments; \**P*<0.05 and \*\**P*<0.005 by unpaired t test. (C) Western blot analysis of HIF1A, HIF2A and HIF1B under hypoxic/normoxic conditions in EKPC and

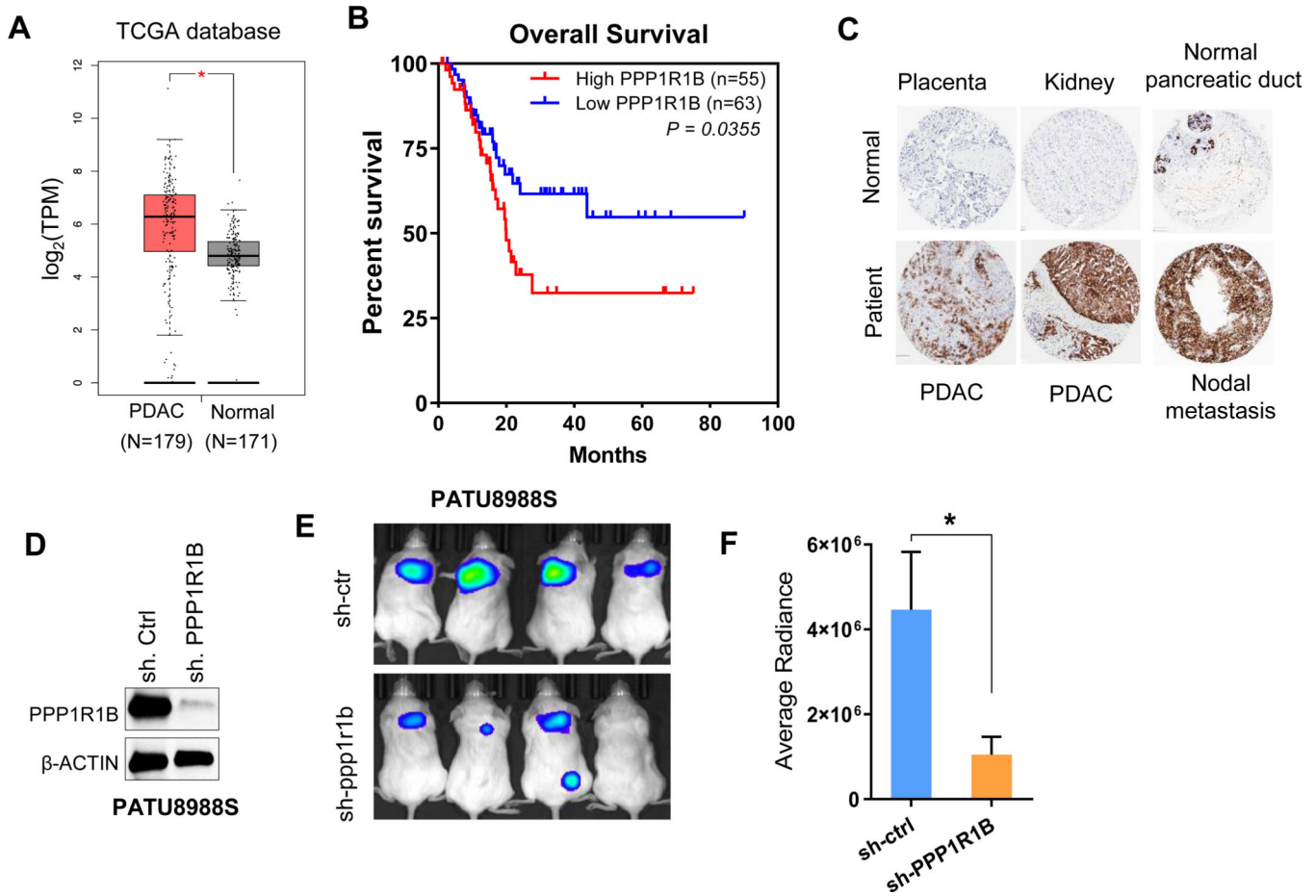
HIF1A<sup>-/-</sup>EKPC cell lines. Beta-actin was used as loading control. (D) Representative images of immunohistochemistry staining for HIF1A and HIF2A in EKPC and HIF1A<sup>-/-</sup>EKPC tumor sections. Scale bar, 50 μm (E) Quantification of HIF1A and HIF2A positive cells per field of vision in EKPC and HIF1A<sup>-/-</sup>EKPC tumor sections. The data are shown as the mean ± s.e.m. *P* values by unpaired t test. ns, not significant, \*\*\*\**P* < 0.0001. (F&G) Representative images and quantitative analysis of transwell migration and matrigel invasion assay of EKPC and HIF1A<sup>-/-</sup>EKPC cells. Total number of cells per field were counted. n=3 biologically independent experiments \*\*\**P* < 0.001, \*\*\*\**P* < 0.0001 by t test. (H) Immunoblot analysis of EMT markers (E-cad, Snail and Slug) and c-Myc protein in EKPC and HIF1A<sup>-/-</sup>EKPC cells. (I) Schematics of *in vivo* lung colonization assay for metastatic burden. EKPC and HIF1A<sup>-/-</sup>EKPC cells expressing Luciferase were injected into the lateral tail vein of immunocompetent C57BL/6 mice (n=5 for each group), and (J&K) Representative bioluminescence imaging and quantitative analysis of lung metastasis by BLI plot \*\**p* < 0.001 by Mann Whitney test. (L) KaplanMeier analysis of mice injected with EKPC and HIF1A<sup>-/-</sup>EKPC cells. \*\**p* < 0.005 by Log-rank (Mantel-Cox) test.



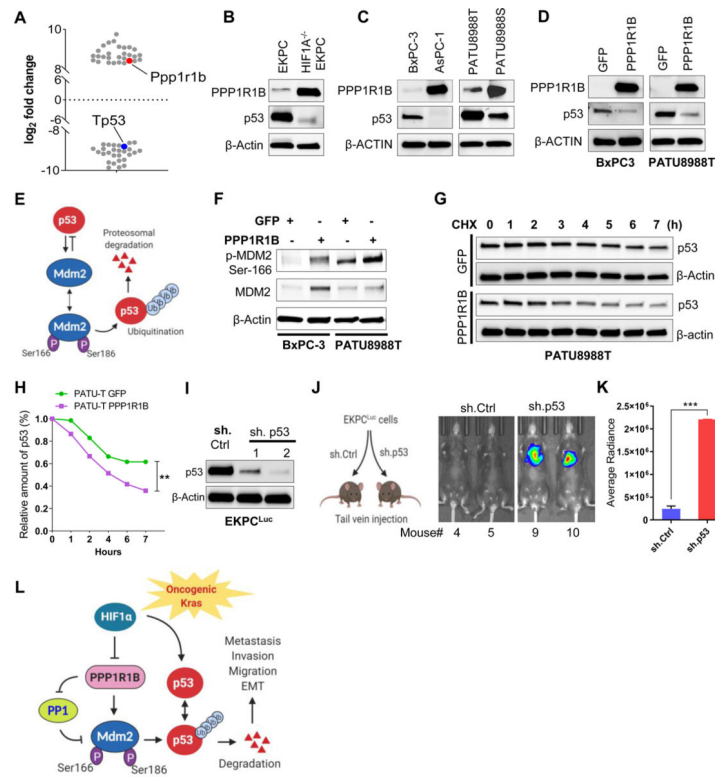
**Figure 3: HIF1A regulates PPP1R1B in pancreatic cancer.**

(A) Schematic workflow of proteomics analysis of EKPC and HIF1A<sup>-/-</sup>EKPC cells (B) The Disease and Function heatmap generated by IPA of the proteomics data reflecting the significantly enhanced (orange) or decreased (blue) cellular functions in HIF1A<sup>-/-</sup>EKPC compared with the EKPC cells. The positive and negative z scores are indicated by orange and blue color respectively. The size of the boxes reflects the predicted direction of change for the function. p values are calculated by Fisher's Exact Test (C) Scatter plot depicting the differentially expressed proteins (DEP) in HIF1A<sup>-/-</sup>EKPC cells compared with the EKPC

cells. Proteins were organized by  $\log_2$  fold change (x-axis) and p-value (y-axis). The cutoff lines (pvalue of 0.05 and  $\log_2$  fold change  $\pm 1.5$ ) are shown in black. PPP1R1B protein is marked exhibiting 8.426  $\log_2$  fold change;  $p=0.016$ . **(D)** qPCR analysis showing Ppp1r1b mRNA levels in EKPC (blue) and HIF1A<sup>-/-</sup>EKPC (orange) cells. Gapdh was used as control. \*\*\* $p < 0.001$  by t test analysis. **(E)** Immunoblot analysis of HIF1A, Ppp1r1b in EKPC and HIF1A<sup>-/-</sup>EKPC cells. **(F)** Western blot analysis of HIF1A and PPP1R1B expression in untreated and CoCl<sub>2</sub> treated (100  $\mu$ M for 12 hr) EKPC and HIF1A<sup>-/-</sup>EKPC cells. **(G,H)** IHC staining and quantification for PPP1R1B<sup>+</sup> cells in EKPC and HIF1A<sup>-/-</sup>EKPC tumors, Scale:200  $\mu$ m, (n = 5 for each group, n = 3 field of vision). \*\*\* $p < 0.001$  by t test analysis. **(I-J)** q-PCR analysis of HIF1A and PPP1R1B transcripts in non/low-metastatic (BxPC-3, PANC-1, Mia PACA-2 and PATU8988T) and high-metastatic (AsPC-1, PATU8988S, SUI-2 and Capan-1) human pancreatic cancer cell lines. \*\*\*\* $p < 0.0001$  by ordinary one-way ANOVA **(K)** Immunoblot analysis of HIF1A and PPP1R1B in non/low-metastatic and high-metastatic human pancreatic cancer cell lines **(L)** Comparative expression analysis of PPP1R1B, HIF1A, HIF1B, HIF2A and HIF3A between non/low-metastatic (BxPC-3, PANC-1, Mia PACA-2 and PATU8988T) and high-metastatic (AsPC-1, PATU8988S, SUI-2 and Capan-1) human pancreatic cancer cell lines. ns, not significant \*\* $p < 0.005$ , \*\*\*\* $p < 0.0001$  by unpaired t test analysis. **(M)** Western blot analysis of PPP1R1B and HIF1A of MIA-PaCa-2 and PATU8988T cells transfected with sh.Ctrl and sh.HIF1A **(N)** Heat map of HIF1A and PPP1R1B expression in PDAC patients from TCGA. **(O)** Paired analysis of PPP1R1B and HIF1A expression in PDAC patients (n=36) ranked based on Z scores ( 0.5). \*\*\*\* $p < 0.0001$  by paired t test. All experiments were carried out in triplicate. All bar graphs represent mean and error bars are s.e.m.



**Figure 4: Increased expression of PPP1R1B correlates with poor survival and its knockdown inhibits malignant properties of PDAC cells**  
 (A). Comparative expression analysis of PPP1R1B in 179 PDAC tissue samples with that of 171 normal pancreas using the TCGA database (GEPID). \* $P < 0.05$  (B) Kaplan–Meier survival analysis of pancreatic cancer patients with high (n=55) and low (n=63) PPP1R1B expression (TCGA), \* $P < 0.05$  by Mantel-Cox test (C) IHC staining of PPP1R1B in human tissue array which includes normal kidney, placenta, benign pancreatic duct, PDACs and nodal metastasis. (D) Western blot analysis of PPP1R1B in PATU8988S cells transfected with sh.Ctrl or sh.PPP1R1B. (E&F) Lung colonization assay of PATU8988S+sh.PPP1R1B and PATU8988S+sh.Ctrl cells in NOD/SCID mice by bioluminescence, n=4 for each group. \* $p < 0.05$  by t test.



**Figure 5: PPP1R1B promotes metastasis in pancreatic cancer by reducing p53**  
 (A) Scatter plot depicting the most significantly up or down regulated proteins ( $> \pm \log_2 8$ ) in HIF1A<sup>-/-</sup>EKPC cells compared with the EKPC cells. Proteins were organized by log<sub>2</sub> fold change (Y-axis). PPP1R1B and p53 are indicated by red and blue respectively. (B,C) Immunoblot analysis of PPP1R1B and p53 levels in mouse and human pancreatic cancer cell lines. β ACTIN was used as control. (D) Western blot analysis of ectopic expression of PPP1R1B and its effect on p53 level in BxPC-3 and PATU8988T cells. GFP was used as control. (E) Schematic model of MDM2 and p53 feedback regulatory loop (F) Western blot analysis of phospho-MDM2<sup>Ser186</sup>, total MDM2 and β ACTIN in BxPC-3 and PATU-8988T cells overexpressing PPP1R1B. GFP was used as control. (G & H) Cycloheximide chase assay and quantification for p53 in PATU8988T cells overexpressing PPP1R1B or GFP. β-ACTIN was used as a loading control. (I) Western blot analysis of p53 and β ACTIN levels in EKPC<sup>Luc</sup> cells stably transfected with sh.Ctrl and two independent sh.p53. (J & K) Lung colonization of EKPC<sup>Luc</sup> + sh.p53 or EKPC<sup>Luc</sup> + sh.Ctrl cells bioluminescence imaging. N=5 for each group. \*\*\**p*<0.0005 by t test (L) Schematic of the mechanistic link between loss of HIF1A, increased expression of PPP1R1B and degradation of the p53 protein that promotes invasion and metastasis in PDAC.



**Table 1:**

Clinical spectrum of disease in HIF1A<sup>-/-</sup>EKPC mice  
 Clinical spectrum of disease in EKPC mice

No.	ID	Genotype	Gender	DOB	DOD	Age (days)	PDA	Liver	Lung	Dia	Lymph	Others	Organs involved in invasion	Total Organ met burden
1	ES33	EKPC Hif1a <sup>fl/fl</sup>	F	11/16/13	04/03/14	138	Y (+++)	Ym	N	N	Y	D <sup>d</sup> ; L <sup>d</sup> ; Adherent to K	2	1
2	ES110	EKPC Hif1a <sup>fl/fl</sup>	F	01/08/14	05/29/14	141	Y (+)	N	N	N	N		0	0
3	ES388	EKPCHif1a <sup>fl/fl</sup>	M	07/14/14	10/24/14	102	Y (+++)	N	N	YM	Y	L <sup>d</sup> ; Adherent to Sp	1	1
4	ES469	EKPC Hif1a <sup>fl/fl</sup>	M	09/13/14	11/18/14	66	Y (+++)	Ym	N	N	Y	Lp <sup>d</sup>	1	1
5	ES593	EKPC Hif1a <sup>fl/fl</sup>	M	12/14/14	03/15/15	91	Y (+++)	Ym	N	YM	Y	D <sup>d</sup> ; L <sup>d</sup>	3	2
6	ES596	EKPC Hif1a <sup>fl/fl</sup>	M	12/14/14	04/28/15	135	Y (+)	N	Ym	N	Y	I <sup>d</sup>	1	1
7	ABA614	EKPCHif1a <sup>fl/fl</sup>	F	01/16/13	08/05/13	199	Y (+++)	YM	YM	YM	Y	D <sup>d</sup> ; S <sup>d</sup> ; I <sup>d</sup>	3	3
8	ES643	EKPC Hif1a <sup>fl/fl</sup>	M	01/06/15	05/15/15	129	Y (+++)	Ym	Ym	Ym	N		0	3
9	ABA618	EKPC Hif1a <sup>fl/fl</sup>	M	01/16/13	05/19/13	123	Y (+++)	Ym	N	YM	Y	D <sup>d</sup> ; K <sup>d</sup> ; GB <sup>d</sup>	3	2
10	ES658	EKPCHif1a <sup>fl/fl</sup>	F	01/24/15	05/22/15	118	Y (+)	N	Ym	N	Y	Sp <sup>m</sup> ; GB <sup>M</sup>	1	3
11	ABA664	EKPC Hif1a <sup>fl/fl</sup>	M	02/16/13	07/25/13	159	Y (+++)	YM	N	N	Y		0	1
12	ES696	EKPC Hif1a <sup>fl/fl</sup>	F	02/14/15	07/24/15	160	Y (++)	Ym	YM	N	N	St <sup>m</sup>	0	3
13	ES717	EKPC Hif1a <sup>fl/fl</sup>	M	03/03/15	05/22/15	80	Y (++)	Ym	YM	Ym	N	D <sup>d</sup> ; I <sup>d</sup>	2	3
14	ABA742	EKPCHif1a <sup>fl/fl</sup>	F	03/17/13	07/09/13	114	Y (+++)	Ym	N	N	Y	D <sup>d</sup> ; S <sup>d</sup> ; Adherent to I	2	1
15	ABA763	EKPC Hif1a <sup>fl/fl</sup>	F	03/06/13	07/25/13	141	Y (+++)	Ym	YM	Ym	Y	D <sup>d</sup> ; L <sup>d</sup> ; Lp <sup>M</sup>	2	4
16	ABA784	EKPC Hif1a <sup>fl/fl</sup>	M	03/25/13	07/04/13	99	Y (++)	Ym	Ym	N	Y	D <sup>d</sup> ; L <sup>d</sup>	2	1
17	ABA 869	EKPCHif1a <sup>fl/fl</sup>	M	04/24/13	08/15/13	113	Y (+++)	YM	N	N	Y	I <sup>d</sup> ; L <sup>d</sup>	2	1
18	ABA909	EKPC Hif1a <sup>fl/fl</sup>	M	05/06/13	09/10/13	127	Y (+++)	YM	N	N	Y	D <sup>d</sup> ; L <sup>d</sup>	2	1
19	ABA933	EKPC Hif1a <sup>fl/fl</sup>	M	05/18/13	08/26/13	100	Y (+++)	Ym	N	YM	Y	D <sup>d</sup> ; L <sup>d</sup>	2	2
20	ABA950	EKPC Hif1a <sup>fl/fl</sup>	M	05/24/13	08/15/13	83	Y (+++)	YM	YM	YM	Y	Sp <sup>m</sup> ; K <sup>m</sup> ; H <sup>m</sup> ; Dia <sup>d</sup> ; L <sup>d</sup> ; D <sup>d</sup> ; L <sup>sp</sup>	4	5
21	ABA954	EKPCHif1a <sup>fl/fl</sup>	M	05/30/13	09/13/13	106	Y (+++)	N	N	N	Y	D <sup>d</sup>	1	0
22	ABA973	EKPC Hif1a <sup>fl/fl</sup>	M	07/01/13	10/17/13	108	Y (+)	Ym	Ym	N	Y		0	3
23	ABA1029	EKPC Hif1a <sup>fl/fl</sup>	F	08/28/13	12/02/13	94	Y (+)	N	Ym	N	Y	L <sup>d</sup>	1	1

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No.	ID	Genotype	Gender	DOB	DOD	Age (days)	PDA	Liver	Lung	Dia	Lymph	Others	Organs involved in invasion	Organs involved in invasion	Total Organ met burden
24	ABA1031	EKPCHfla <sub>nr/nr</sub>	M	08/28/13	10/08/13	41	Y(+++)	N	YM	N	Y	St <sup>d</sup>		1	1
1	ES149	EKPC	F	02/05/14	07/30/14	175	Y(+++)	YM	YM	YM	Y	D <sup>d</sup> ; L <sup>d</sup> ; Sp <sup>d</sup>	3		3
2	ES154	EKPC	M	02/05/14	10/29/14	177	Y(++)	N	N	N	Y		0		0
3	ES236	EKPC	M	03/24/14	05/29/14	66	Y(+)	Ym	N	N	N		0		1
4	ES273	EKPC	M	04/19/14	10/14/14	175	Y(++)	N	N	N	N		0		0
5	ES368	EKPC	M	07/05/14	02/08/15	213	Y(++)	N	N	N	Y	D <sup>d</sup>	1		0
6	ABA336	EKPC	M	08/16/12	12/12/12	116	Y(+++)	N	N	N	N		0		0
7	ABA405	EKPC	F	10/15/12	05/13/13	210	Y(+)	Ym	N	YM	Y		0		2
8	ABA407	EKPC	F	10/15/12	04/08/13	175	Y(+)	YM	N	N	N		0		1
9	ABA541	EKPC	F	12/10/12	03/17/13	97	Y(+)	N	N	N	N		0		0
10	ABA567	EKPC	F	12/27/12	09/06/13	249	Y(++)	YM	N	N	Y		0		1
11	ABA571	EKPC	M	12/27/12	07/25/13	208	Y(++)	N	YM	N	N		0		1
12	ABA642	EKPC	F	02/11/13	05/01/13	79	Y(+++)	Ym	N	N	N	I <sup>d</sup>	1		1
13	ABA775	EKPC	M	03/24/13	08/06/13	135	Y(+)	YM	N	N	Y		0		1
14	ABA806	EKPC	F	04/06/13	07/12/13	97	Y(++)	Ym	N	N	Y		1		1
15	PP451	KPC	M	01/10/14	08/20/14	222	Y(+++)	N	Ym	N	N		0		1
16	PP509	KPC	M	02/01/14	09/30/14	241	Y(+++)	N	N	N	Y		0		0
17	PP793	KPC	M	04/19/14	08/28/14	131	Y(+++)	N	Ym	N	N	L <sup>d</sup>	1		1
18	PP866	KPC	M	05/16/14	10/22/14	159	Y(++)	Ym	Ym	N	N	L <sup>d</sup>	1		2
19	PP901	KPC	F	05/24/14	09/18/14	117	Y(+++)	N	N	N	Y		0		0
20	PP1011	KPC	F	06/19/14	12/19/14	183	Y(+++)	N	Ym	N	Y		0		2
21	PP1012	KPC	F	06/19/14	10/03/14	106	Y(+)	N	N	N	N		0		0
22	PP1034	KPC	M	06/21/14	12/21/14	183	Y(+++)	YM	Ym	Ym	Y		0		3
23	PP1041	KPC	M	06/27/14	09/10/14	75	Y(+)	N	N	N	N		0		0
24	PP1051	KPC	F	06/30/14	10/03/14	95	Y(++)	N	N	N	N		0		0
25	PP1091	KPC	F	08/13/14	11/13/14	90	Y(+++)	N	N	Ym	N		0		1

X, disease detected [ >50% normal acini (+), 20–50% normal acini (++) and <20 % normal acini (+++)]}; N, no disease detected; M, macrometastasis; m, micrometastasis; d, direct invasion; Ab, abdominal wall; L, liver; Lp, lymph node, I, intestine; Sp, spleen; St, stomach; GB, gall bladder; K, kidney; D, Duodenum

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